METHOD OF STUDYING THE FUNCTIONAL STATE OF PULMONARY ALVEOLAR MACROPHAGES UPON EXPOSURE TO ATMOSPHERIC POLLUTANTS

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A method of evaluating the functional state of the pulmonary alveolar macrophages of rabbits and rats as one criterion of the biological effect of chemical environmental pollutants based on the results of experimental investigations is proposed. The method described includes a cytological investigation, a determination of the number of viable cells, and the phagocytic activity, as well as a biochemical study of the activity of various enzymes of the alveolar macrophages (acid phosphatase, β-glucuronidase, lysozyme, β-galactosidase, β-glucosidase, N-acetyl-β-D-glucosaminidase). It is shown that the method proposed is informative and reliably reproducible and can be used in studying questions of environmental health and in other areas of experimental biology and medicine.

Pulmonary alveolar macrophages (PAM) largely determine the resistance of the organism to the inhalation of various unfavorable environmental factors [6, 10]. This phenomenon is due to the biological function of the PAM, which is manifested in phagocytic activity [3, 13], participation in processes of cellular immunity, the production of interferon [5], etc. The development and improvement of methods of studying the functional state of the PAM as one criterion of the biological effect of atmospheric pollutants acquires great importance in connection with this.

Our work in this area* has led to the development of a complex method of studying the functional activity of the PAM, which includes a cytological and biochemical investigation of alveolar macrophages isolated from the lungs.

The method of isolating alveolar macrophages from the lungs is equally applicable to experiments both with rats and with rabbits. The animals are sacrificed by the administration of nembutal (for rats 100 mg/kg intraperitoneally, for rabbits 150 mg/kg into the ear vein), a tracheotomy is performed, and physiological solution is introduced into the lungs through a probe inserted into the trachea with the help of a syringe (5 ml for rats and 30 ml for rabbits). After 15 min approximately half of the physiological solution introduced is drawn out of the lungs with a syringe into a centrifuge tube, after which the above-indicated amount of physiological solution is introduced five times, drawing it off each time and placing all the liquid collected into one tube. The wash liquid obtained, whose final volume constitutes 25-30 ml for rats and 150-200 ml for rabbits, is a suspension of alveolar macrophages, polymorphonuclear neutrophils (PN), lymphyoctes, erythrocytes, and other cells located on the surface of the pulmonary alveoli. In intact animals PAM predominate, constituting 97-99% of the total number of cellular elements [8].

Cytological Investigation. A cytological investigation is conducted after centrifugation of the washing liquid for 15 min at 1500 rpm and resuspension of the sediment obtained in 2 ml of physiological solution.

Determination of Total Number of Cells. The total number of cells in the suspension is counted in a celloscope.

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Percentage Content of Alveolar Macrophages. The percentage content of alveolar macrophages and other cellular elements (primarily PN) in the suspension is determined by means of immersion microscopy of a smear treated in the following way: staining with hematoxylin-eosin for 30 min, decolorization in 95% ethanol, fixation for 2-3 min in 100% ethanol, and two washings in xylene.

Counting of Viable PAM. The number of viable PAM in the suspension is counted by means of light microscopy of a smear of the suspension stained with 0.4% trypan blue [11].

Determination of Phagocytic Activity of PAM. The phagocytic activity of the PAM is determined after removal of the other cells contained in the suspension. To purify the PAM 2 ml of the suspension is layered on 6 ml of 25% bovine albumin with a density gradient of 1.062, pH 7.4 [12], and centrifuged for 15 min at 850 rpm at −2°C. The upper layer of the liquid containing the PAM is drawn off with a Pasteur pipet, washed twice with 2 ml of physiological solution, followed by centrifugation for 15 min at 1500 rpm and −2°, and the sediment is separated from the supernatant.

Determination of Phagocytic Activity. The phagocytic activity is determined in a suspension of purified PAM. To 3 ml of a suspension containing $2 \cdot 10^6$-$3 \cdot 10^6$ cells/mm^3 is added 0.1 ml of a suspension of plastic particles (suspension of polystyrene latex spheres, Dow Diagnostics, USA), the number of which should be 100 times greater than that of the PAM. The mixture is incubated for 60 min in water bath at 37°, after which smears are made, fixed for 12 h in vapors of a 40% solution of formalin, and then stained with hematoxylin-eosin.

The number of phagocytic macrophages and the phagocytic index is determined by means of immersion microscopy [9].

Biochemical Investigation of Activity of Enzymes of PAM. The biochemical activity of the enzymes of the PAM is investigated after the removal of other cellular elements from the suspension of PAM. If the content of the former, especially of PN, does not exceed 10-15%, the sediment is washed with 2 ml of an 0.85% solution of ammonium chloride, which leads to lysis of the erythrocytes and stabilizes the activity of the enzymes [14].

The sediment of PAM is then washed twice with physiological solution, followed by centrifugation of the suspension for 15 min at 15,000 rpm and −2°, and removal of the supernatant. The sediment obtained is resuspended in 1 ml of physiological solution and the activity of the enzymes is determined in the suspension.

If the amount of PN in the suspension of PAM exceeds 10-15%, purification is conducted by a method analogous to that used in determining the phagocytic activity of the macrophages.

Determination of Acid Phosphatase Activity. To 0.1 ml of a suspension of ultrasonically treated PAM is added 0.1 ml of an 8 mM solution of the disodium salt of p-nitrophenyl phosphate (Sigma, USA) in 0.1 M acetate buffer solution containing 2 mM magnesium chloride (pH 5.0) and 0.1% Triton X-100. The amount of p-nitrophenol released is determined spectrophotometrically (410 nm) after 30 min of incubation in a water bath at 37°, followed by cooling and the addition of 2.5 ml of 0.25 M NaOH. The standard used is 0.1 ml of 1 mM p-nitrophenol (Eastman Organic Chemicals, Rochester, N.Y.). The activity of the enzyme is expressed in micromoles of p-nitrophenol per minute per milligrams of protein.

Determination of β-Glucuronidase Activity. The β-glucuronidase activity is determined by a previously described method [7]. To 0.25 ml of a suspension of PAM treated with an 0.1% solution of Tritox X-100 is added 1 ml of 0.1 M acetate buffer solution (pH 4.5) and 0.25 ml of an 0.01 M solution of phenolphthalein glucuronic acid (pH 7.0), which is used as the substrate (Sigma, USA). After 60-minute incubation in a water bath at 37° the reaction is stopped by the addition of 1 ml of 95% ethanol, followed by filtration of the sample through a paper filter. To 1 ml of the transparent filtrate is added 1 ml of glycine buffer solution (pH 10.0) and the pink solution is subjected to spectrophotometry at 545 nm. The activity of phenolphthalein released in 1 min per milligram protein.

Lysozyme Activity. To 2.5 ml of 0.15 M phosphate buffer solution (pH 6.2), prepared in 0.9% physiological solution and containing 0.25 mg/ml Micrococcus lysodeikticus is added 0.5 ml of a suspension of ultrasonically treated PAM. After 30 and 60 sec the reaction mixture is subjected to spectrophotometery at 450 nm and the activity of the enzyme is determined from the difference in optical densities and expressed in arbitrary units per milligram protein.

The β-galactosidase and β-glucosidase activities are determined using a macrovariation of these methods [4].

Determination of β-Galarosidase Activity. To 0.4 ml of a suspension of PAM is added 0.4 ml of a 0.4 ml of a 5 mM solution of the substrate, p-nitrophenyl-β-D-galatopyranoside, prepared in 0.1 M citrate–phosphate buffer solution (pH 5.0), and 0.6 ml of this buffer. The sample is incubated for 30 min at 37°. The reaction is stopped by the addition of 1.6 ml of 0.4 M glycine buffer (pH 10.8).

β-Glucosidase Activity. The β-glucosidase activity is determined in an incubation mixture consisting of 0.4 ml of a suspension of PAM, 0.4 ml of substrate (5 mM p-nitrophenol-β-D-glucopyranoside), prepared in 0.1 M citrate–phosphate buffer solution (pH 5.0), and 0.6 ml of this buffer containing 0.1% Triton X-100. The sample is incubated at 37° for 30 min, after which the reaction is stopped by the addition of 1.6 ml of 0.4 M glycine buffer (pH 10.8).

N-Acetyl-β-D-hexosaminidase Activity. The N-acetyl-β-D-hexosaminidase activity is determined by a method published earlier [1]. A suspension of PAM (0.5 ml) is mixed with 1 ml of 0.1 M phosphate–citrate buffer solution (pH 4.4) containing 500 μg of the substrate, p-nitrophenyl-β-D-glucosaminide, and incubated for 60 min at 37°. After incubation 1.2 ml of 0.2 M borate buffer solution (pH 9.8) is added to the samples.

The β-galactosidase, β-glucosidase, and N-acetyl-β-D-hexosaminidase activity in the PAM after centrifugation of the samples at 8000 rpm for 15 min is determined spectrophotometrically at 420 nm from the p-nitrophenol released and expressed in micromoles of p-nitrophenol per milligram protein in 1 min. The substrates used for determining the activity of these enzymes in the present work were prepared by Chemapol (Czechoslovalia).

The functional state of the PAM was studied in an experiment on rabbits and rats (a total of 200 animals) exposed to atmospheric pollutants.

The results of the present work and previous investigations performed during the inhalation of nitrogen peroxide, ozones, and aerosols of metals, some of which were presented in previous publications [8, 15], showed the informativeness and reliable reproducibility of the proposed method. For example, as a result of a joint experiment the dependence of the changes in the various enzyme systems of rat PAM on the dose and times of the constant inhalation effect of NO_2 (14 and 28 ppm) was established. This dependence was manifested primarily in an ambiguous direction of the disturbances in the activity of the PAM enzymes, namely: in a decrease in the β-glucouronidase (β-glu) and acid phosphatase (AP) activity of the PAM in specific periods of the action of the higher concentration of NO_2 (28 ppm), which was not observed at the lower dose of this substance (14 ppm). In the latter case in different periods of the investigation either a tendency toward an increase in the activity of the enzymes of rat PAM or the absence of specific changes as compared to the control group of rats was characteristic.

The fact that a statistically significant decrease in the β-glu activity of rat PAM (by 31%, $P < 0.05$) was observed in an earlier period of the action of the higher concentration of NO_2 (after 0.2 h) as compared to that of AP, in which only a tendency toward a decrease in activity was observed in this period, is of interest. However, it should be noted that in later periods of the experiments (after 1.6 and especially after 24 h at high dose of NO_2) the AP activity of rat PAM, in contrast to that of β-glu, showed a statistically significant decrease as compared to its values in the control rats.

The dissimilar direction and degree of disturbance in the activity of acid hydrolases of rat PAM in different periods of the action of different concentrations of NO_2 that we found may be due to phases of the development of adaptation mechanisms and to a change in the functional state of the PAM under the action of NO_2. It is also possible that inhibition of the *de novo* synthesis of enzyme proteins is one of the causes of the decrease in the activity of the enzyme systems of the PAM studied under the influence of the inhalation effect of chemical substances in general and NO_2 in particular. Considering the fact that AP is among the hydrolases bound to membranes on the lysosomes, while β-glu can have both a lysosomal and a microsomal origin, it can be assumed that the decrease that we observed in the activity of these enzymes is due to a disturbance in the function of the membranes of various subcellular structures of the PAM. A more detailed study of the metabolic mechanisms of the change in the activity of enzymes of the PAM under the influence of atmospheric pollutants will be the subject of our further investigations in this area.

Thus, the cytological and biochemical methods of evaluating the functional state of rat PAM proposed in the present work can be used in a study of questions of environmental health as well as in other areas of experimental biology and medicine.

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